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Identification of a Missense Mutation and Several Polymorphisms in the Proenkephalin A Gene of Schizophrenic Patients

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Schizophrenia is a complex and severe disorder of unknown cause and pathophysiology. In this study, we examined the opioid hypothesis for schizophrenia at the molecular level, focusing on the dopamine-regulated proenkephalin A gene (chromosome 8q11.23-q12). We have screened 150 schizophrenic patients for sequence variations within the promoter region, entire coding sequence, and 3′-untranslated region. We find one sequence change in a conserved amino acid that may be of functional significance. This mutation was found in a single schizophrenia patient but not in controls. Although several new, race-specific polymorphisms were identified, all other sequence changes appeared to be common polymorphisms, unlikely to contribute to the etiology of schizophrenia.

KEY WORDS: schizophrenia, genetics, candidate gene, opioid

INTRODUCTION

Schizophrenia is a severe disorder characterized by psychotic symptoms such as delusions and hallucinations and/or debilitating behavioral symptoms such as deficits of emotion, mental activity, and social drive [Carpenter and Buchanan, 1994]. Often beginning in early adulthood, the effects of the disease typically result in lifelong disability in social and occupational functions. Schizophrenia afflicts approximately 1% of the population worldwide, making this one of the most widespread diseases known. Schizophrenia occurs at approximately the same prevalence across diverse geographic, cultural, and socioeconomic categories [Carpenter and Buchanan, 1994].

Despite intense research efforts due to the severity and prevalence of the disease, its biochemical basis is still not understood. Likewise, the importance of genetic factors vs. environmental factors in schizophrenia has been a matter of debate. Family, adoption, and twin studies do support a genetic component for schizophrenia [McGuffin et al., 1995; Kendler and Diehl, 1993; Kendler et al., 1985; Lowing et al., 1983]. However, single-gene Mendelian transmission is not evident, as indicated by twin studies showing that the rate of concordance (extent to which both members of pairs of twins either do or do not express a trait) for dizygotic twins is approximately 10% and that for monozygotic twins is 40–50% [McGuffin et al., 1995; Kendler, 1983; Onstad et al., 1991]. A multifactorial etiology (with environmental as well as genetic factors) is likely, but the number and identity of genes involved is unknown.

Several approaches have been undertaken to elucidate the genetic component of schizophrenia, including linkage analysis [Su et al., 1993], linkage disequilibrium-based association studies [Nanko et al., 1994], and candidate gene-based association studies [Sobell et al., 1993]. As pharmacologic and other data have suggested dopaminergic involvement in schizophrenia [Creese et al., 1976; Peroutka and Snyder, 1980; Seeman et al., 1976], many genes selected as candidates for association studies have been components of dopamine pathways. However, no mutations in dopamine receptors have been clearly associated with disease [Sarkar et al., 1991; Sommer et al., 1993; Yang et al., 1993; Gejman et al., 1994]. This lack of evidence has directed efforts towards examination of defects in dopamine metabolism and/or signal transduction within dopamine pathways including the interaction of dopamine with other neurotransmitter systems. One of these is the opioid system.

Opioid peptides are a family of neuropeptides which derive from three precursor gene products: proenkephalin A [PENK] [Noda et al., 1982], proopiomelanocortin [POMC] [Nakanishi et al., 1979], and proenkephalin B [prodynorphin] [Kakidani et al.,

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1982]. Opioids are neuromodulators which influence a wide variety of neurological functions including pain control, autonomic reflexes, and behavioral control [Van Nispen et al., 1990]. Similarities between the pharmacological effects of opioids and psychotic symptoms of psychiatric illnesses led to hypotheses that these peptides are involved in the pathophysiology of schizophrenia [Wiegant et al., 1992]. However, because the different opioid genes produce many bioactive peptides with overlapping products and functions, studies measuring opioid peptide levels in schizophrenic patients and controls, as well as trials of opioid peptides and receptor antagonists in schizophrenia treatment, are variable, difficult to interpret, and, therefore, inconclusive [Nemeroff and Bissette, 1988].

In this study, we examine the opioid hypothesis for schizophrenia at the molecular level. We have begun our analysis with the proenkephalin gene because, in basal ganglia circuits, enkephalin peptides coexist with γ-aminobutyric acid (GABA) in medium spiny projection neurons of the striato-pallidal pathway where proenkephalin expression is modulated by dopamine [Angulo, 1992; Chen et al., 1994; Tang et al., 1983]. We have screened 150 schizophrenic patients for sequence variations within the promoter region, entire coding sequence (including intron-exon boundaries), and 3′-untranslated region. We detected several new and common polymorphisms within the proenkephalin gene that vary by race and may be useful as markers for linkage studies, as well as one mutation of possible functional significance which warrants further evaluation for association with schizophrenia.

MATERIALS AND METHODS

Patients

One hundred fifty schizophrenic patients and forty-nine control individuals were chosen for study from a large collection of cases and controls described by Sobell et al. [1993]. All schizophrenic cases were diagnosed by a research psychiatrist (L. Heston, M.D.) based on DSM-III-R criteria [Am. Psych. Assoc., 1987]. Strict fulfillment of the diagnostic criteria was assessed primarily through the review of medical records. The overwhelming majority of patients were chronic schizophrenics who were ascertained as research volunteers while in state hospitals. Lengthy, detailed medical records, including archived records, were available for most patients [Sobell et al., 1993]. The patients included 115 Caucasians of northern or western European descent, 25 African-Americans, 5 Asians, and 5 Native Americans. Controls were Caucasian and African-American. DNA was extracted from peripheral blood as previously described [Gustafson et al., 1987].

Oligonucleotide Synthesis

Oligonucleotide primers for polymerase chain reaction (PCR) and sequencing were synthesized using standard solid state synthesis techniques by the Mayo Foundation Molecular Biology Core Facility. Primers were purified by passage through Bio-Rad Econo-Pac 10DG disposable 10 ml desalting columns, lyophilized, resuspended in sterile water, and stored at −20°C.

PCR Conditions

PCR reactions were performed using primers listed in Table I. The 20 μl reactions contained 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 8.8), 0.1% Triton X-100), $MgSO_4$ to concentrations listed in Table I, 0.1 μM each primer, 0.2 mM each dNTP, approximately 0.1 μg genomic DNA, 0.2 μl α-^{32}P[dCTP] (3,000 Ci/mmol, ICN, Costa Mesa, CA), and 0.4 U Deep-Vent DNA Polymerase (exo-) (New England Biolabs, Beverly, MA). Cycling conditions were: 98°C for 3 min, followed by 30 cycles of 98°C for 1 min, annealing temperature (Table I) for 5 sec, 75°C for 30 sec, with a final extension at 75°C for 10 min.

Single-Strand Conformation Polymorphism (SSCP) Analysis

SSCP analysis [Orita et al., 1989] was carried out as described by Glavac and Dean [Glavac and Dean, 1993]. A 4 μl aliquot of labeled PCR product was mixed with 8 μl loading buffer (80% formamide, 10 mM NaOH, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1% each of bromophenol blue and xylene cyanol dyes). The samples were denatured at 95°C for 3 min and rapidly cooled on ice. Two microliters of each sample were loaded (using a sharkstooth comb) onto SSCP gels (33 × 40 cm) prepared from 8% acrylamide (37.5:1 acrylamide:bis) and Tris-Borate EDTA buffer (89 mM Tris base, 89 mM boric acid, and 2 mM EDTA). Electrophoresis was carried out with a sequencing gel apparatus (CBS Scientific, Del Mar, CA) run at 45 W constant power for 5–10 hr in a cold room. Gels were allowed to cool for 45 min in the cold room prior to use and prerun for 15 min. After electrophoresis, gels were dried and auto-radiographed for 48–72 hr at −70°C.

DNA Sequencing

Genomic DNA was amplified by PCR as described above, with the exception that radiolabel was excluded from the reaction. For sequencing any region of exon 3, primers I2(−32)-20D and 3′UT (+335)-18U were used to generate a 1 kb PCR product. PCR products were purified (to remove excess primers and nucleotides) with Microcon-100 microconcentrators (Amicon, Beverly, MA) according to product instructions. PCR products were then sequenced by Circum Vent Thermal Cycle Dideoxy DNA Sequencing (New England Biolabs) as described in the instruction manual. Deoxy/dideoxy sequencing mix concentrations used were those recommended for Deep-Vent (exo-) DNA polymerase. Annealing temperatures used were those listed in Table I for PCR. Primers were end-labeled using T4 polynucleotide kinase (New England Biolabs) with [γ-^{32}P]-ATP (7,000 Ci/mmol, ICN).

Dideoxy Fingerprinting (ddF)

Genomic DNA was amplified by PCR, the PCR products were transcribed by T7 or Sp6 RNA polymerase, and the resulting RNA was used as template for

TABLE I. Primers and Conditions for Amplification of Proenkephalin Gene Regions*

Downstream primer	Upstream primer	PCR prod.	Anneal. temp. (°C)	$MgSO_4$ conc. (mM)
1D = AGGCGTCGGCGCG	1U = GGCGTCGGGGAGAA	243 bp	52	2
2D = CCGCAGAGACGCTG	2U = GCAGACGTCCCCAAA	232	50	2
3D = GCTCGAAGCACGCG	3U = GTCTCACAAGGTGCG	213	50	4
4D = CTTTTTATCATTACATCAAA	4U = GGCTAGCAAATGGC	185	44	8
5D = AAACCGGAAGAAAGCC	5U = GAATTGGCCAGCGA	195	46	4
D_1 = T7-AATTCCTCAGACCTGC	C_2 = Sp6-CTTCCTCATTATCACTG	134	60, 30 s.	6
6D = ACAACCGAGAGCGT	6U = TCCATACCGTTTCTGG	200	48	4
7D = AAGAGTAGGTCGCCCA	7U = GAGGCTTGCTGGGG	200	50	6
8D = TTCCCACTAGTGGCC CCAGGC	8U = CGAGATAGCTGCAATAGA CTAATACTGAGC	215	58	8
9D = AAGCTCAGTATTAGTCT	9D = AAAGAGACTTACAAGGAT	160	44	8
PCR primers for ddF[a]:				
A_1 = T7-CCGACCCCTCCCG	A_2 = Sp6-AATGTGAAGGGAAAGAG	500	55	2
B_1 = T7-TTGCTCGACGATCCC	B_2 = Sp6-CGGAAACTCTGTCTCA	300	55	2
C_1 = T7-TTCTTTTTATCATTACATCA	C_2 = Sp6-CTTCCTCATTATCACTG	500	55	2
D_1 = T7-AATTCCTCAGACCTGC	D_2 = Sp6-GAGCCTTGGAGAAAAA	750	55	2
Sequencing primers for ddF (additional)				
aD = CGTTCTGCGCCCAG	fU = AAACCGAAGGAGGCG			
bD = TCATGAAGAAGGATGCA	gU = AGCTCCTTGCAGGTTT			
cD = CCCAACTGGAAGATGA	hU = CCATGGGATAAAGCTC			
dD = TCCAAAGAAGTTCCTGA	iU = ATCCTGGTGGTGGCT			
eD = CTGAAAACTGTCATTTCA	jU = AGATAGCTGCAATAGAC			

*All primer sequences are written 5′ → 3′.
[a]RNA polymerase promoter sequences attached to primers were (5′ → 3′):
T7 = TAATACGACTCACTATAGGGAGA
Sp6 = ATTTAGGTGACACTATAGAATAG.

reverse-transcriptase sequencing reactions (one base only, ddT in this study). Reactions were separated in non-denaturing sequencing gels to look for changes in banding patterns. PCR reactions were performed using primers listed in Table I, containing Sp6 or T7 polymerase promoters. The 20-μl reactions contained: 50 mM KCl, 10 mM Tris, pH 8.4, 2.0 mM $MgCl_2$ 0.1 μg genomic DNA, 0.2 μM each primer, 0.2 mM each dNTP, and 0.5 U Taq DNA polymerase. Amplification with primers A_1 and A_2 was carried out as a nested reaction using a 1:100 dilution of A_1-B_2 PCR product. Cycling conditions were: 94°C for 3 min, followed by 30 cycles of 94°C for 1.5 min, 55°C for 2 min, and 72°C for 2.5 min, with a final extension at 72°C for 10 min. PCR products were transcribed by Sp6 or T7 RNA polymerase and transcripts used in ddF reactions as described by Sarkar et al. [1992], where ddT was the dideoxy nucleotide used and electrophoresis was carried out with a Bio-Rad sequencing apparatus.

Allele Frequency Determination

Allele frequencies were calculated as a percentage: number of a specific allele/total number of alleles × 100, where number of a specific allele is the sum of subjects homozygous for that allele × 2 and subjects heterozygous for that allele × 1, and total number of alleles is the total number of subjects × 2.

RESULTS

The PENK gene was screened for single base-pair changes by either dideoxy fingerprinting (ddF) or single-strand conformation polymorphism (SSCP) analysis. In ddF, the products of a dideoxy sequencing reaction (using only one chain terminator) are separated on a nondenaturing gel, producing a ladder of termination products. PCR and sequencing primers used for this method are shown in Figure 1A (also see Table I). For SSCP analysis, PCR products (with an average size of 200 base pairs, ranging from 134 to 243 base pairs; see Table I and Fig. 1C) were loaded directly onto nondenaturing gels for electrophoresis as described in Materials and Methods.

Figure 1B shows a representative ddF result. The reactions were performed using the downstream primer 2D, with T7 transcripts of A_1/A_2 PCR products used as templates. As seen by the different banding patterns, this gel demonstrates a common sequence polymorphism. Sequencing identified this polymorphism as a C →A base change in the untranslated exon 1 [E1(98)]. Lane 1 contains a sample homozygous for the C at this position, lane 2 contains a sample homozygous for the A, and lane 3 contains a heterozygote.

Figure 1d represents a typical SSCP result. PCR products were obtained from primers 3D and 3U, amplifying exon 2 of the PENK gene. This gel clearly demonstrates a polymorphism, as seen on the top strand. Sequencing revealed a C → T silent base change in Ser 27 of exon 2 (AGC → AGT). Lane 1 contains a sample homozygous for the C in Ser 27, lane 2 contains a sample homozygous for the T, and lane 3 contains a heterozygote.

A total of 150 schizophrenic patients were screened for mutations. Approximately 20% of patient samples were analyzed by both ddF and SSCP, with the excep-

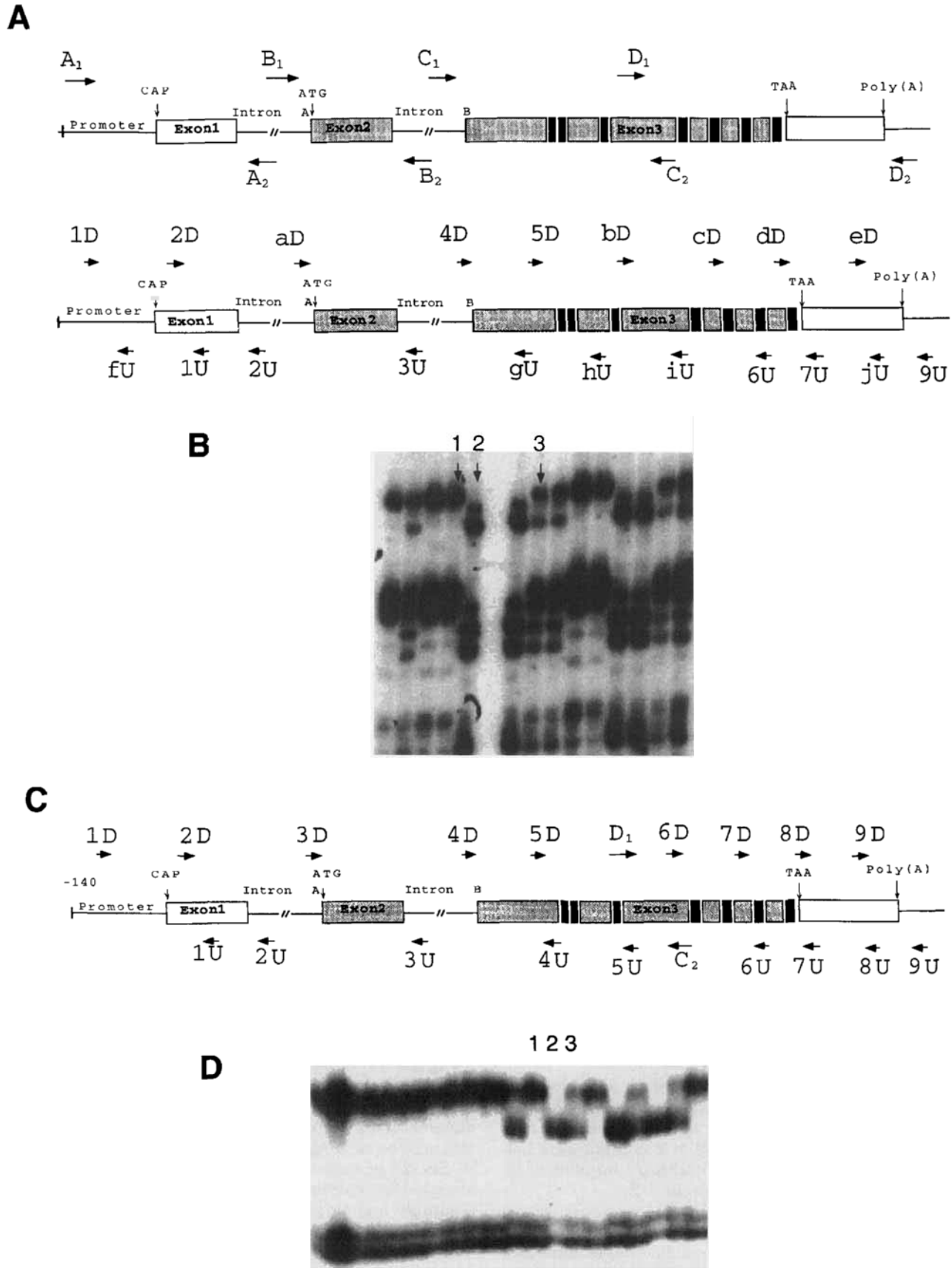

A
A1
B1
C1
D1
CAP
ATG
TAA
Poly(A)
Promoter
Exon1
Intron
Exon2
Exon3
A2
B2
C2
D2
1D
2D
aD
4D
5D
bD
cD
dD
eD
fU
1U
2U
3U
gU
hU
iU
6U
7U
jU
9U
B
1 2 3
C
-140
3D
D1
6D
7D
8D
9D
4U
5U
8U
D
1 2 3

Fig. 1.

tion of the final 150 base pairs of the coding region, which were screened only by SSCP. Although some ddF analyses were suboptimal, the ability of two independent techniques to yield identical results suggest that the data are accurate. The remaining samples were analyzed by SSCP alone. The patient set consisted of 115 Caucasians, 25 African-Americans, 5 Native Americans, and 5 Asians. We detected a total of six polymorphisms (see Fig. 2A). Three of these predominated in Caucasian subjects: C →A[E1(98)] (Fig. 1B), a C deletion in the 3′-untranslated region close to the stop codon [3′UTR(+7)], and an A →G base change near the polyadenylation consensus sequence in the 3′UTR [3′UTR(+319)]. The C →A[E1(98)] and C del[3′UTR-(+7)] polymorphisms were generally inherited together (were very tightly linked) and occurred with allele frequencies of 49% in Caucasians. The A →G[3′UTR-(+319)] polymorphism was found in 30% of Caucasian patients. The three remaining polymorphisms appeared primarily or exclusively in the African-American population. These were all C →T silent base changes in coding regions of the gene: Ser 27(AGC → AGT) (Fig. 1D), Ser 243(TCC →TCT), and Tyr 261(TAC →TAT). The Ser 243 and Tyr 261 polymorphisms also appear to be linked: of the 25 African-American patient samples, one patient was homozygous for both changes and three patients were heterozygous for both changes. A set of 28 Caucasian control subjects and a set of 21 African-American control subjects were screened by SSCP for these polymorphisms, with allele frequencies very similar to those of the respective schizophrenic patient groups. Table II lists allele frequencies by racial group.

In the one African-American patient who was homozygous for the two C →T silent changes in Exon 3, sequencing revealed an additional base change. This patient was heterozygous for a G →A mutation in Exon 3 which results in the substitution of aspartic acid for glycine at amino acid 247 (see Fig. 3). The original African-American patient (n = 25) and control (n = 21) sets were expanded and all samples were sequenced in this region. In a total of 46 African-American schizophrenic patients and 40 African-American controls, this mutation was detected in only the one patient, resulting in an allele frequency of 1.1% for patients and 0% for controls (Fig. 3).

DISCUSSION

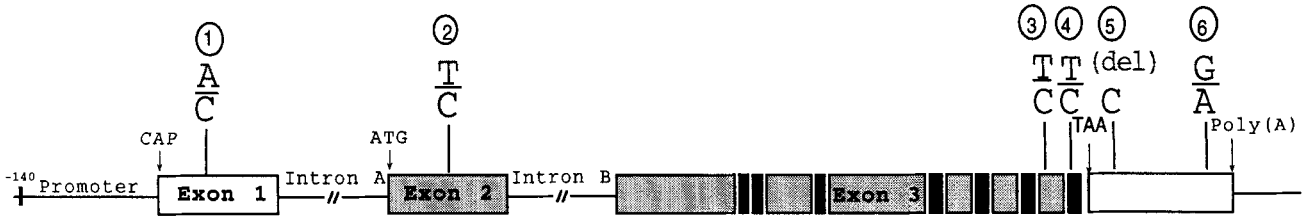
The purpose of this study was to test proenkephalin A as a candidate gene for involvement in the genetic transmission of schizophrenia. This gene was screened for mutations of likely functional significance in a total of 150 schizophrenic patients. Although the gene was not screened in its entirety (introns were omitted), we examined regions of likely regulatory function (such as the promoter/5′-untranslated region and 3′-untranslated region), coding regions, and intron/exon boundaries.

Three polymorphisms were detected primarily in Caucasians. These three polymorphisms all occurred in many patients (higher frequencies than would be expected for mutations making small contributions to a complex disease), in non-coding regions, and did not generate cryptic splice sites, making any link between these sequence changes and disease unlikely. In addition, the C → A[E1(98)] polymorphism occurred in a region of exon 1 not conserved among different species. The C del[3′UTR(+7)] and A → G[3′UTR(+319)] polymorphisms, however, did occur in more highly conserved sequences which, being near the sites of translation termination and polyadenylation, could have effects on regulatory or processing events. When a control set of Caucasian subjects was screened for the three polymorphisms, however, comparable allele frequencies were obtained. The data suggest that the polymorphisms found in the proenkephalin A gene are not likely to contribute to disease, and allele frequencies reported for this patient set are representative of the general population. Interestingly, the E1(98) and the 3′UTR polymorphisms appear linked in the Caucasian and Native American populations, but not in the African-American population.

In a set of non-Caucasian patients, an additional three polymorphisms were detected in the coding regions of the gene (see Table II). Although the Tyr 261 polymorphism did occur in a region encoding an enkephalin peptide, all three changes were silent (did not change the encoded amino acid) and did not generate cryptic splice sites. In addition, the frequency of codon usage was not significantly altered by the silent changes (for Tyr, codon TAC is used 60% while TAT is used 40%; for Ser, TCC usage vs. TCT is 23.5% vs. 18% and AGC vs. AGT is 25% vs. 13% [Ellington and Cherry, 1995]). Therefore, these polymorphisms also are not likely to be of functional significance. Two of these polymorphisms, the Ser 243 and Tyr 261 silent changes, are linked and occurred exclusively in the African-

Fig. 1. Screening for proenkephalin mutations. **A:** Schematic representation of the human proenkephalin gene indicating promoter, exons, introns, transcription start site (CAP), translation start site (ATG), and the polyadenylation site [Poly (A)]. Exons are indicated as boxes; introns are represented as lines (not to scale). The shaded area represents the translated region. The black boxes are the enkephalin peptides. The indicated regions of the gene were amplified by PCR in four regions, using primer pairs A_1/A_2, B_1/B_2, C_1/C_2, and D_1/D_2. T7 transcripts were used as templates for sequencing and ddF reactions with downstream sequencing primers (1D-eD); Sp6 transcripts were used as template with upstream sequencing primers (fU-9U). All ddF reactions were performed using ddT as the chain terminator. **B**: A representative portion of a ddF gel, where products were separated on a 6% polyacrylamide gel. These reactions were carried out with [γ-^{32}P] end-labeled primer 2D covering the second half of Exon 1. **Lane 1** illustrates the banding pattern for a common polymorphism from an individual homozygous for the expected sequence (C at position 98); **lane 2** illustrates the banding pattern for an individual homozygote for a C→A base change; and **lane 3** contains a heterozygote. **C**: A schematic representation of the proenkephalin gene (as described in A), indicating the location of PCR primer pairs used for SSCP. Primers were spaced such that each SSCP segment was approximately 200 base pairs (see Table I). Products were radiolabeled by incorporation of α-^{32}P-dCTP and electrophoresed on non-denaturing 8% polyacrylamide gels. **D**: A representative banding pattern detected by SSCP analysis of Exon 2 utilizing primers 3D/3U. **Lane 1** contains a homozygote for a common polymorphism; **lane 2** contains a homozygote for a sequence change (C→T silent change in Ser 27), and **lane 3** contains a heterozygote.

A



B

	Name	Sequence context	Location
1	C->A E1(98)	CAGCACACC**C**GGGC 98	untranslated Exon 1
2	C->T E2(Ser27)	TGC AG**C** CAG Cys Ser Gln 26 27 28	Exon 2 Silent change
3	C->T E3(Ser243)	CCC TC**C** GAC Pro Ser Asp 242 243 244	Exon 3 Silent change
4	C->T E3(Tyr261)	AGA TA**C** GGC Arg Tyr Gly 260 261 262	Exon 3 - Enk. peptide Silent change
5	C (del) 3'UTR(+7)	TAATATCTT +7	3'-untranslated region Near stop codon
6	A->G 3'UTR(+319)	AATAAACCT**A**TT +319	3'-untranslated region Near polyadenylation consensus sequence

Fig. 2. Polymorphisms in the proenkephalin gene. **A**: A schematic representation of the proenkephalin gene (as described in the legend to Fig. 1), summarizing the locations of the six common sequence polymorphisms detected in the gene. **B**: A more detailed description of the sequence context and relevance of location of the polymorphisms.

American population. Also, the Ser 27 silent change was found predominantly in the African-American population. When a set of control African-American subjects was screened for all six polymorphisms, results were very similar to the African-American patient set, so that these allele frequencies appear representative of the general African-American population.

One mutation of possible functional significance was discovered. The Gly (247) → Asp mutation was detected in only one from a total of 46 African-American patients and was not found in any of a comparable number of African-American controls. This amino acid is completely conserved among species (human, bovine, rat, mouse, and frog) suggesting the potential for functional significance. The proenkephalin A precursor protein is highly processed to small bioactive peptides [Docherty and Steiner, 1982]. Although the mutation is not found within a Met-enkephalin (Met-enk) processed peptide, it is found within a highly conserved enkephalin-containing peptide (ECP) called peptide B whose function is unknown [Stern et al., 1981; Kilpatrick et al., 1981; Lindberg and White, 1986; Watkinson et al., 1989]. Further, the alteration from a non-polar (Gly) to a charged amino acid (Asp) and its location between two nearby enkephalin peptides suggests that the replacement could influence processing/cleavage events. Further screening will be necessary to establish association with schizophrenia.

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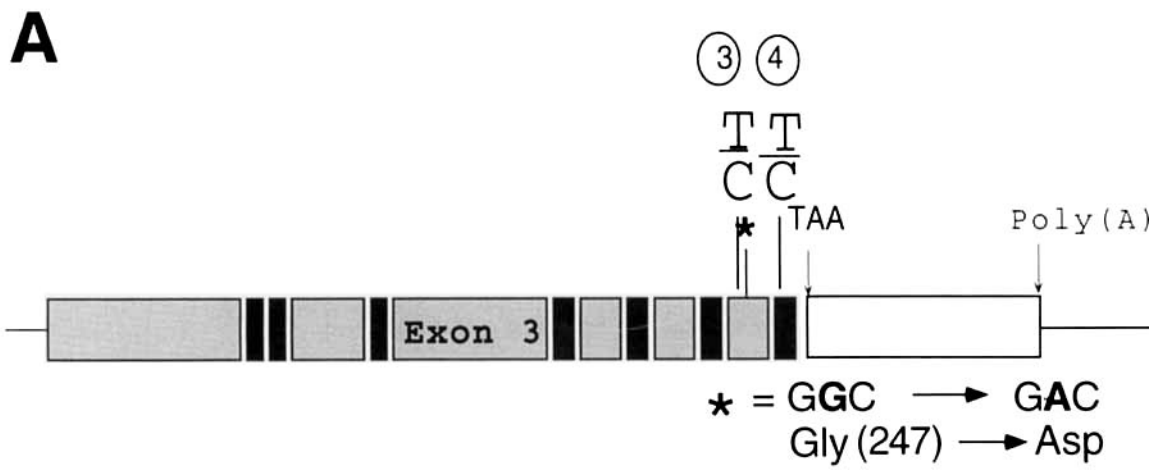
TABLE II. Allele Frequencies of PENK Polymorphisms*

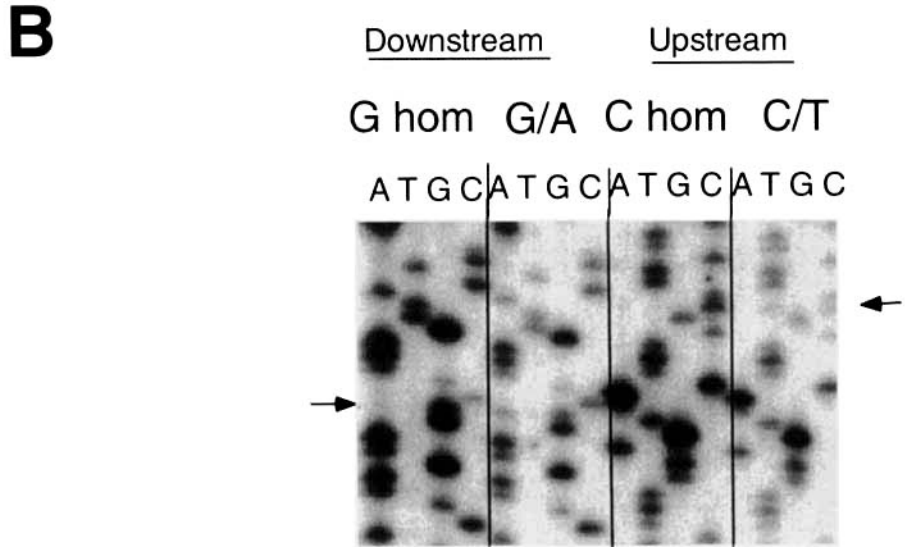
Ethnicity	(n) Sample size	Frequency of polymorphic allele: E1 (98) C→A	3′UTR C del (d)	3′UTR A→G	Ser 27 C→T	Ser243 C→T	Tyr261 C→T
Caucasian	115	.49	.49	.30	.009	0	0
Caucasian control	28	.39	.39	.37	—	0	0
African-American	25	.14	.10	.10	.38	.10	.10
Afr.-Am. control	21	.26	.19	.095	.24	.071	.071
Native American	5	[.40][a]	[.40]	[.30]	[.20]	[0]	[0]
Asian	5	[.40]	[.40]	[.30]	[0]	[0]	[0]

		Genotype distribution (n): CC	CA	AA	CC	Cd	dd	AA	AG	GG	CC	CT	TT	CC	CT	TT	CC	CT	TT
Caucasian	115	31	55	29	31	55	29	58	44	13	113	2	0	115	0	0	115	0	0
Caucasian control	28	12	10	6	12	10	6	12	11	5	—	—	—	28	0	0	28	0	0
African-American	25	19	5	1	20	5	0	20	5	0	12	7	6	21	3	1	21	3	1
Afr.-Am. control	21	12	7	2	15	4	2	17	4	0	14	4	3	18	3	0	18	3	0
Native American	5	2	2	1	2	2	1	3	1	1	3	2	0	5	0	0	5	0	0
Asian	5	2	2	1	2	2	1	3	1	1	5	0	0	5	0	0	5	0	0

*Allele frequencies for schizophrenic patients and control subjects were calculated by racial group. The sample size (n) listed for each group is the number of subjects screened, so the total number of alleles used in the frequency calculation was 2 × n. The genotype distribution indicates the number of subjects (n) possessing each genotype (homozygous for the more common sequence, heterozygous, homozygous for the polymorphism). The number of alleles of a particular polymorphism was calculated as the sum of n (het.) and 2 × n (hom. for polymorphism). This number was divided by the total number of alleles in a given subject group to give the allele frequency of each polymorphism. Dashes in the table indicate that a subject set was not screened for a given polymorphism.

[a]Brackets indicate that allele frequencies are not statistically significant because of small sample sizes and reflect only the frequencies of those alleles found in those particular sample sets. More meaningful are the actual numbers of alleles detected, which are listed in the genotype distribution columns.





C

	Sample size (n)	# of individuals with mut.	Allele frequency
Patients	46	1 (het.)	.011
Controls	40	0	0

Fig. 3. Detection of a sequence change of potential functional significance in the proenkephalin coding sequence. **A**: A schematic representation of the 3′ region of the proenkephalin gene (described in the legend to Fig. 1), where an asterisk marks the location of the G→A mutation. The locations of the two silent C→T polymorphisms (3 and 4) in this region are also shown for comparison. **B**: Direct sequencing of the G→A mutation. The schizophrenic patient is a G/A heterozygote; a control subject (G homozygote) is shown for comparison. The base change can be seen in sequencing from both directions and has been confirmed by automated sequencing (not shown). **C**: Expanded screening for the G→A mutation in the African-American population. A total of 46 schizophrenic patients and 40 control subjects (including the original 25 patients and 21 controls) were screened for the G→A mutation by automated sequencing. Allele frequency was calculated as described for Table II.

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